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(54) Title: ASSAY METHODS

(57) Abstract

The present invention relates to an automated format for the yeast two hybrid assay for protein-protein interactions.

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ASSAY METHODS

FIELD OF THE INVENTION

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The present invention relates to methods useful for detecting protein-protein interactions. Protein-protein interactions enable the association of two or more proteins through the formation of non-covalent bonds when two protein surfaces are precisely matched. These bonds account for the specificity of recognition. Protein-protein
10 interactions are involved, for example, in the assembly of enzyme subunits; in antigen-antibody reactions; in forming the supramolecular structures of ribosomes, filaments, and viruses; in transport; and in the interaction of receptors on a cell with growth factors and hormones. Products of oncogenes can give rise to neoplastic transformation through protein-protein interactions.

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BACKGROUND OF THE INVENTION

The yeast two-hybrid (Y2H) assay is a method for detecting protein-protein interactions using a genetic system. The technique may be used for charting protein
20 interactions, and hence, identifying potential partners in genetic pathways. The assay is sensitive and yields the DNA sequences encoding proteins that interact. In a typical two-hybrid assay, a known protein that forms part of a DNA-binding domain hybrid is assayed against a library of all possible proteins present as transcriptional activation domain hybrids. Some two hybrid approaches rely on interaction mating. In this method, the
25 protein fused to the DNA-binding domain and the protein fused to the activation domain are expressed in two different haploid yeast strains of opposite mating type and the strains are mated to determine if the two proteins interact. When haploid yeast strains of opposite mating type come into contact, mating occurs and results in fusion of the two

haploids to form a diploid yeast strain. An interaction can thus be determined by measuring activation of a two-hybrid reporter gene in the diploid strain.

WO 94/10300 and U.S. Patent No. 5,283,173 describe methods for detecting the
5 interaction between proteins using reconstitution of the activity of a transcriptional
activator. This reconstitution makes use of chimeric genes which express hybrid proteins.
The first hybrid contains the DNA-binding domain of a transcriptional activator fused to a
known protein (the "bait"), with the DNA binding domain DNA binding element placed
upstream of a reporter gene. "Prey" proteins are cloned as either random sequences or
10 cDNAs and are fused to the amino- or carboxy-terminus of a transcription activation
domain. If the bait and prey proteins are able to interact, they bring into close proximity
the two domains of the transcriptional activator. This proximity is sufficient to cause
transcription, which can be detected by the activity of a reporter gene that contains a
binding site for the DNA-binding domain.

15

The disadvantages of these techniques is that irrelevant interactions with yeast
proteins are generated. These include false-positive interactions that are unlikely to be
found in living cells, and false-negative interactions, that is, those interactions that would
otherwise be detected but are not. The techniques as disclosed in WO 94/10300 and U.S.
20 Patent No. 5,283,173 require the use of mating in solid medium which is cumbersome,
labor-intensive, and does not preserve diploid cells for further analysis.

We have developed the mating strategy of the yeast two-hybrid assay into an
automated format which allows many bait proteins to be processed. The format uses an
25 arraying means, for example, microtiter plates and liquid mass-mating of a subset of a
large, complex library. By tracking positive interactions in the library, we have also
developed a method to create a functionally-subtracted library, that is, one that can be
made devoid of a scorable phenotype. For example, our method allows for the

determination of detection of hybrids that react promiscuously with many targets, such as heat shock proteins, and their elimination from any future considerations.

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SUMMARY OF THE INVENTION

In accordance with the present invention there is provided a method for detecting protein-protein interactions comprising liquid mass-mating of subsets of a large, complex library. The method provides a means for subtracting irrelevant protein-protein
10 interactions to yield a "functionally-subtracted" assay.

DETAILED DESCRIPTION OF THE INVENTION

15 According to an aspect of the present invention, there is provided a method for detecting an interaction between a first test protein and a second test protein, comprising:

(a) providing a host cell containing a reporter gene wherein the reporter gene expresses a detectable protein when the reporter gene is activated by an amino acid
20 sequence including a transcriptional activation domain when the transcriptional activation domain is in sufficient proximity to the reporter gene;

(b) providing a first chimeric gene that is capable of being expressed in the host cell, the first chimeric gene comprising a DNA sequence that encodes a first hybrid protein, the first hybrid protein comprising:

25 (i) a DNA-binding domain that recognizes a binding site on the reporter gene in the host cell; and

(ii) a first test protein or fragment thereof that is to be tested for interaction with at least one second test protein or fragment thereof;

(c) providing a second chimeric gene that is capable of being expressed in the host cell, the second chimeric gene comprising a DNA sequence that encodes a second hybrid protein, the second hybrid protein comprising:

(i) the transcriptional activation domain; and

5 (ii) a second test protein or fragment thereof that is to be tested for interaction between the first test protein or fragment thereof; wherein interaction between the first test protein and the second test protein in the host cell causes the transcriptional activation domain to activate transcription of the reporter gene;

(d) introducing the second chimeric gene into the host cell and subsequently
10 introducing said cells into an arraying means thereby creating a master library plate;

(e) introducing cells from the master library plate into a second arraying means thereby creating a mating set;

(f) introducing the first chimeric gene into the host cell and subsequently introducing said cell into the mating set;

15 (g) selecting for outgrowth of the interaction of the first and second genes;

(h) removing a portion of the mating set to a third arraying means thereby creating a rescue set;

(i) determining whether the reporter gene has been expressed in the mating set;

and

20 (j) analyzing the cells from the rescue plate.

The term "reporter gene" or "marker gene" as used herein means any gene whose expression may be assayed. More than one reporter gene may be encoded by the host cell in step (a) above.

25

The term "arraying means" as used herein means any method for holding clones in liquid media, suspension, or solid media, for example, microtiter plates or test tubes.

The term "selecting for outgrowth" as used herein means any method using a selectable means to either amplify or isolate a set of interacting proteins. This selectable means may include outgrowth in a nutritionally-deficient growth medium wherein the interacting proteins cause transcription of a biosynthetic gene or pathway. Examples of other useful selectable means include amino acid, metabolic, catabolic and nucleic acid biosynthetic genes, such as yeast HIS3, URA3, and LYS2, GAL1, *E. coli* galK, and CAT, GUS, antibiotic resistance, and any gene encoding a cell surface antigen for which antibodies are available. Outgrowth may be allowed to proceed for 5 - 10 days prior to selecting for outgrowth.

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The term "analyzing" as used herein means any method for obtaining information regarding protein-protein interactions, for example, selecting positive clones, performing PCR, DNA sequence analysis, and comparison with databases such as LifeSeq® (Incyte Pharmaceuticals) or Genbank.

15

The term "functionally substracted" means devoid of a detectable phenotype that represents an irrelevant protein-protein interaction.

In a further aspect of the invention, determination of reporter gene expression and analysis of cells may be accomplished in one step, that is steps (i) and (j) above may be combined. Alternatively, steps (h), (i), and (j) may be eliminated.

A eukaryotic host strain, for example, a yeast strain, may be engineered to express the protein (the "bait") of therapeutic or diagnostic interest as a fusion protein covalently bonded to a known DNA binding domain of a transcriptional activator. The eukaryotic host strain also contains one or more "reporter genes", that is genes whose transcription is detected in response to a bait-prey interaction. Bait proteins, via their DNA binding domain, bind to their specific DNA site upstream of a reporter gene; reporter gene

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transcription is not stimulated, however, because the bait protein lacks its own activation domain.

To isolate genes encoding novel interacting proteins, cells of this strain containing
5 a reporter gene and expressing a bait protein are transformed with individual members of
a DNA (for example, a cDNA) expression library. Each member of the library directs the
synthesis of a candidate interacting protein fused to a weak and invariant gene activation
domain tag. The library-encoded proteins ("prey" proteins) that physically interact with
the promoter-bound bait protein detectably activate the transcription of the downstream
10 reporter gene and provide a ready assay for identifying particular cells which harbor a DNA
clone encoding an interacting protein of interest.

In one embodiment, a cDNA library, created in *E. coli*, and comprising cDNA fused
to the DNA sequence encoding the activation domain of the transcriptional activator,
15 GAL4 protein, is plated onto 960 LB agar plates at a density of 1000 clones per plate. The
E. coli colonies on each plate are pooled, plasmid DNAs are isolated, and the DNAs are used
to transform yeast. The transformed yeast are plated onto solid medium and the colonies
on each plate are pooled and aliquoted to separate wells of a 96-well microtiter plate to
create an arrayed set of 10 "master library" plates. Five microliters from each well of the
20 master library set is re-aliquoted to create a "mating set" and 5 μ l of bait-containing yeast
is then added separately to each well. The "bait" comprises a chimeric gene that expresses
a hybrid protein containing the DNA-binding domain of GAL4 fused to a known protein.
The host yeast strain contains the GAL1-lac-Z gene, which is able to bind the GAL4 DNA-
binding domain. The GAL1-lacZ gene contains the *E. coli* lacZ gene encoding β -
25 galactosidase. The activity of β -galactosidase is a measure of GAL4 function. Growth of
yeast on galactose requires the transcription of genes regulated by GAL4 and is also a
measure of GAL4 function. The liquid mass-mating is allowed to proceed for a period of
time and the mating mixture is diluted 100 fold with leucine drop-out medium. After
outgrowth of positively-interacting mated yeast diploids in the drop-out medium, a

portion is removed to a separate set of "rescue" plates and a β Gal analysis is performed on the mating set. Transcriptional activation can be determined by measuring β -galactosidase activity on galactose containing media. Wells containing any β Gal activity are identified and clones from the corresponding set of wells from the rescue plates are analyzed by PCR
5 sequencing.

In another aspect of the invention, there is provided a method for producing a functionally subtracted master library. Irrelevant protein-protein interactions may be eliminated by recombining only productive clones to the master library, thereby
10 eliminating clones that produce proteins that are known to interact with many other proteins, for example heat shock-proteins.

In another further aspect of the present invention, there is provided a method for an open reading frame cloning strategy that involves the dynamic recoding of the ends of
15 DNA molecules. This cloning strategy increases the efficiency of the assay by eliminating from analysis all clones that encode proteins that are out of frame with respect to the activation domain.

In dynamic recoding of an activation domain, the 3' end of the activation domain
20 gene may be recoded to incorporate an amino acid hybrid peptide that also encodes the DNA controlling elements necessary for *E.coli* gene expression. In one aspect, these controlling elements comprise, in series; i) a sequence, for example, -35 and a -10 sequence, which acts as an *E.coli* promoter to initiate mRNA transcription, ii) a ribosome binding site and an ATG fMet codon necessary to initiate protein translation, iii) a multiple
25 cloning site composed of one or more restriction sites which are preferably unique to the cloning vector, into which to clone stuffer fragments of DNA that can encode protein fusions to the activation domain, and iv) a reporter gene, for example, the lacZ gene, cloned out-of frame with respect to the ATG codon. In the open reading frame cloning system, the ATG may be in-frame with respect to the activation domain, the ATG may be

out-of-frame with respect to the lacZ gene, there is a negligible amount of β Gal protein produced by the host cell in the absence of a stuffer fragment that restores the lacZ gene reading frame, and there is an absence of termination codons the end of the activation domain gene and the ATG codon.

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The term "stuffer fragments" means any fragment of DNA generated synthetically, or through use of a method generally used to generate random or 3'-end primed cDNA molecules that can be cloned into the multiple cloning site of the above open reading frame cloning system.

10

In one aspect, random primed cDNA used as stuffer fragments may be sized-selected by agarose or polyacrylamide gel electrophoresis. Individual cDNA sized-selected by gel electrophoresis or other means may contain fragments which when cloned into the vector system described may be in one of six reading frames (3 reading frames in both forward and reverse orientation).

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The recoded activation domain may be used in conjunction with the out-of-frame reporter gene to select for clones that restore the reading frame of the reporter gene. For example, if the lacZ gene is initially out-of-frame with respect to the ATG start of the recoded portion of the activation domain, then clones that restore the reading frame between that ATG and the lacZ gene will create protein fusions of that clone to the lacZ gene product. Fusions that restore β gal activity may be selected for chromogenically by using well-known dyes (e.g., Xgal) or on selective growth media containing lactose as the sole carbon source.

20

In a further aspect of the open reading frame cloning system, an *E.coli* suppressible termination codon (for example a TAG amber termination codon) may be encoded between the stuffer fragment and the reporter gene such that in phenotypically suppressing *E.coli* host strains the stop codon is suppressed by a suppressing tRNA molecule that inserts a

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specific amino acid. In non-suppressing host cells in which the interaction assay is performed the termination of protein translation would occur at the termination codon. The advantage to having this suppressible system is that the open reading frame reporter protein will not be fused to the carboxyl terminus of the encoded stuffer fragment-
5 activation domain hybrid protein.

In another aspect of the invention there is provided the method according to steps (a) through (g) above or (a) through (i) above in the form of a kit for rapid screening.

10 The host cell may be any type of cell, including yeast, bacterial, or mammalian cell. The preferred host cell is a yeast cell, advantageously *Saccharomyces cerevisiae*.

The bait protein may be derived from a bacterial protein, a viral protein, an oncogene-encoded protein, a growth factor or an enzyme. Bait proteins may be chosen
15 from any protein of known or suspected diagnostic or therapeutic importance. Preferred bait proteins include oncoproteins (such as myc, ras, src, fos) or any other proteins involved in cell cycle regulation (such as kinases, phosphatases).

Prey proteins may be encoded on a library of plasmids containing DNA inserts that
20 are derived from genomic DNA, cDNA, or synthetically generated DNA sequences fused to the DNA sequence encoding the second amino acid domain. cDNAs may be constructed from any mRNA population and inserted into an equivalent expression vector. Such a library of choice may be constructed *de novo* using commercially available kits (for example, from Stratagene, La Jolla, CA) or using well established preparative procedures
25 (for example, Current Protocols in Molecular Biology, New York, John Wiley & Sons, 1987). Alternatively, a commercially available cDNA library may be used. A prey protein may be encoded by a synthetic sequence or may be the product of a randomly generated open reading frame or a portion thereof.

Any suitable reporter gene may be used, for example, LEU2 gene or the lacZ gene. Examples of other useful genes whose transcription can be detected include amino acid and nucleic acid biosynthetic genes, such as yeast HIS3, URA3, and LYS2, GAL1, *E. coli* galK, GFP, CUP1, and CAT, GUS, antibiotic resistance, and any gene encoding a cell surface
5 antigen for which antibodies are available.

Those skilled in the art will also recognize that the reporter gene, DNA binding domain, and gene activation domain components may be derived from any appropriate eukaryotic or prokaryotic cell genomes or cDNAs as well as artificial sequences. Moreover,
10 although yeast represents a preferred host organism, other host organisms such as mammalian cells may also be utilized.

Plasmid constructs, transformation, transfection, cell culture and detection of transcription may be performed by any method known in the art, for example, U.S. Patent
15 No. 5,283,173 and WO 94/10300, incorporated herein by reference hereto.

Any means for introducing genes into host cells may be used, for example, electroporation, transfection, transformation, or mating.

20 Advantages of the described invention include increased efficiency through elimination from further analysis of promiscuous proteins in arrayed libraries, creation of a means to functionally subtract classes of proteins from libraries, elimination from further analysis of clones not in a specified reading frame, reduced labor over current methods, re-use of primary libraries from arrayed master library sets, and accumulated knowledge
25 over time of the make-up of the arrayed clones.

The invention may be illustrated by the following non-limiting examples.

EXAMPLE 1Liquid mass-mating, functionally-subtracted yeast two-hybrid assay

5 Restriction and DNA modification enzymes were purchased from various manufacturer's and used according to their recommendations.

 Creation of arrayed cDNA libraries (Figure 1). *E.coli* cDNA libraries were purchased from Invitrogen and were plated at a low density (approximately 1000 clones per plate)
10 onto LB + Amp plates and incubated 1-2 days at 37 °C. Next, 3-4 ml of LB (containing 15% glycerol) was added to each plate, the plate rocked on a platform shaker at low speed, and the LB harvested after resuspension of the colonies in the LB was apparent. A 200 µl portion of the resuspended cells was removed for plasmid DNA isolation and the remaining cells frozen for long-term archival storage at -80 °C.

15 Plasmid DNA was isolated by means of a kit obtained from Qiagen. Two hundred fifty (250) µl of P2 solution (Qiagen) was added to the 200 µl portion of cells in a 2 ml Eppendorf centrifuge tube. The two solutions were mixed gently and then 250 µl P3 solution (Qiagen) was added and the tube shaken. The mixture was then centrifuged
20 at high speed (14,000 rpm) in an Eppendorf centrifuge. The clarified supernatant (500 µl) was pipetted to a new Eppendorf centrifuge tube and 1 ml of ethanol added to precipitate the DNA. The precipitated DNA was pelleted at high speed (14,000 rpm) for 15 minutes, the ethanol solution decanted off, and the pellet dried *in vacuo*. The pellet was resuspended in 50 µl distilled H₂O and used directly to transform the yeast.

25 Yeast were transformed using the EZ Yeast Transformation kit (Zymo Research) according to the manufacturer's recommendation, using 2.5 µl DNA, 25 µl competent yeast strain EGY48 and 250 µl of EZ3. The transformed yeast were incubated for 1 hr at 30 °C and the total plated onto SD - trp agar plates. The plates were incubated for an

additional 3-4 days at 30 °C, and the cells harvested as for *E.coli* using 3-4 ml SD - trp + 15% glycerol. The harvested yeast from each plate were separately aliquoted into different wells of a deep-dish 96-well plates (the "master library" plates) and frozen at -80 °C for long-term storage.

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Yeast liquid-mating (Figure 2). Five μ l from each of the yeast Master Library well was inoculated into 100 μ l of SD - trp or SGal-trp in a 96-well plate and grown overnight at 30 °C. Five μ l of each well were transferred to a new 96 well "mating" plate. A 5 μ l aliquot of a bait culture ($OD_{600} = 1.0$) was added to each well along with 10 μ l YPD medium. The mating plates were placed into a resealable plastic bag and incubated for 12-36 hr at 30 °C. Each well was then twice serially-diluted 10-fold (final 100 fold dilution) using S-min (- leu, - his, - trp, - ura, + gal, + raff) to a final volume of 110 μ l. Alternatively, each well was then diluted 1:10 into S-min, incubated at 30° C for two days, and then diluted 1:40 in S-min (final 400 fold dilution). The diluted matings were incubated for an additional 5-10 days at 30 °C. Ten μ l of the mated wells were then transferred to a second set of plates prior to performing the β Gal analysis (these mated and out-grown 10 μ l stocks ("rescue plates") were later used for rescuing positive clones).

β Gal Assay. Cells were lysed by the addition of 100 μ l of a solution of Z buffer [Na₂HPO₄, (16.1 g l⁻¹), NaH₂PO₄, (5.5 g l⁻¹), KCl (0.75 g l⁻¹), and MgSO₄, (0.25 g l⁻¹), adjusted to pH 7.0 and sterile-autoclaved] containing oxalyticase (100 U ml⁻¹), SDS (0.1%), and CPRG substrate (2 mg ml⁻¹). The plates were incubated at room temperature until the red β Gal chromogenic substrate developed (usually 10 min to 2 hr). To quantitatively measure the wells it was necessary to remove the cell debris by either centrifugation or filtration. The CPRG substrate may be measured at an absorbance of 575 angstrom. Alternatively, β -gal activity was measured using a chemiluminescent substrate. The Tropix Galacton Plus kit was used for this purpose. Twenty-five microliters from each assay well were transferred to the corresponding wells of 96-well luminescence plates. Twenty-five microliters of CL Reaction Buffer (Z buffer containing 0.2% Igepal CA-630, 100 U/ml

oxalyticase, and 1% Galacton Plus) was added to each well, and the plates were incubated overnight at room temperature. Fifty microliters of Accelerator II (diluted 1:1 in 0.1M Na₂CO₃/NaHCO₃, pH 10.5) was added to each well, and the plates were incubated for 5 minutes. Chemiluminescence was then measured in a 96-well luminometer.

5

Test of pooling sensitivity. A test of the pooled liquid mating strategy was performed using the known strong Y2H interactors RPB4 (yeast polII subunit) and RPB7 (yeast polII subunit) as controls. The RPB4 subunit was subcloned into the activation-domain vector pJG4.5. The recombinant RPB4 fusion was subcloned into the DNA binding domain vector pEG202, transformed into the prey strain and mixed at various percentages (from 0 to 100%) with the same prey strain containing the pJG4.5 parental vector.

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The results (shown in Figure 3) demonstrated that we were able to recover prey strain for this interaction even when the prey initially represented approximately 0.1% of the prey "mating mixture." The results suggested that dilution of the complex YPD medium approximately 100-fold may be needed in order to see differential growth of the positively-interacting pairs. Dilution of the samples to lower the concentration of YPD complex may be preferable to other methods, such as centrifugation or filtration. This is because dilution is cheaper, faster, and easier to automate. The β Gal analysis of the test of the reporter activation in a pooled microtiter plate format showed no significant difference between 0.1 and 100% recombinant fusion at the 100-fold dilution point. At higher dilutions scattering of the β Gal activity occurred. It may be that at the higher dilutions (of low percentage pools) sampling of positive interactors may be lost.

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Test of pooled arrayed cDNA libraries. In the first test of the arrayed-cDNA library experiment, the nuclear receptors RXR and LXRA were tested against ca. 6×10^5 cDNAs in 6 microtiter plates. Most of the cDNAs were from the commercially-available cDNA libraries derived from human fetal liver (Invitrogen A202-01) and human fetal brain (Invitrogen A212-01).

The cDNA-containing clones were seeded at approximately 1×10^3 clones per well. Briefly, the bait strain (containing the target protein, in this case either RXR or LXR α) was added to the cDNA library clones in the wells and mating was allowed to proceed in a complex medium. The mated mixtures were diluted into minimal medium (-leucine) and growth of interactors allowed to take place (growth indicating successful interaction) over 5 or more days. β Gal assays were then performed on the wells (see example, Figure 2), and clones from 10 wells exhibiting substantial β Gal activity were re-isolated by streaking an aliquot of the library well onto solid minimal medium (-leucine). Plasmids were isolated from those clones and subjected to DNA sequence and bioinformatics analysis. The results are shown below in Table I.

Some of the sequenced clones have been found through traditional Y2H analysis. These include TRIP6 (thyroid receptor interacting protein 6) which has been previously described in the literature from other "standard" interaction-trap experiments against other Nuclear Receptors (it had not yet been tested against LXR α) and TIF1. We believe these represent true-positives. The other clones, both encoding a GCN5 homolog, were isolated twice (in two different wells). We do not yet know if GCN5 homolog is a true or false positive.

Approximately a third of the interacting clones were found to have homology to cDNAs in the Incyte or GenBank databases, but have no ascribed function.

Several clones appear to be known promiscuous positives in interaction trap experiments (namely, cofilin and the heat shock proteins). Now that we know which wells these are in means that they can be eliminated from future analysis. However, it should be noted that when we do exclude these wells, we are also losing information in about the other clones in that well. For example, using RXR as a bait, we found an interaction with thymopoietin-related protein in well 1D9. Yet this same well, when queried with LXR α

found a positive interaction with the promiscuous positive HSP90. It is hoped that a large enough library of cDNA is eventually used to obtain redundancy in the library analysis.

Table 1. Results of Y2H Analysis

Bait	Sequence ID, well #	Representative Homology	Comments
RXR	5rxr, 1A10	TIF1	known NR (+) ^c
LXRa	13gor4, 3G4	TRIP6	known NR (+) ^c
RXR	1rxr, 1G6	annexin (IPP)	found in standard screen ^c
LXRa	7gor4, 5A10	GCN5 homolog	found in standard screen ^c
LXRa	20gor4, 5C5	GCN5 homolog	involved in transcription ^d
RXR	3rxr, 1D9	thymopoiten-related	involved in transcription
LXRa	12gor4, 2G1	KIAA0229	plausable positive
			Genbank EST,
			(no known function)
LXRa	5gor4, 1H8	Incyte 3122030 ^b	no annotation in GenBank,
			see also 21gor4
LXRa	9gor4, 2F8	Incyte 004215 ^b	no annotation in GenBank
LXRa	15gor4, 4C2	Incyte 1366945 ^b	no annotation in GenBank
LXRa	21gor4, 2B10	Incyte 3122030 ^b	no annotation in GenBank
RXR	7rxr, 3B8	nucleolin gene	known false (+) ^a
LXRa	1gor4, 1A8	huHSP86	known false (+) ^a
LXRa	3gor4, 1D9	huHSP90	known false (+) ^a
LXRa	11gor4, 5E11	cofilin	nvolved in cell structure,
			interacts with actin

^a known common positive in other Y2H screens (E.Golemis).

^b No annotation found to genbank database-specific sequence.

^c This protein is known to interact with several other nuclear receptors.

^d GCN5 possesses histone acetyltransferase (HAT) activity.

^e Protein was also isolated using traditional two-hybrid methodology.

Example 2

Open Reading Frame Cloning Strategy

Cloning open reading frames and use of suppression to control 3' gene fusion

Randomly sheared cDNA of approximately 600 base pairs was isolated and cloned into a frameshifted β gal gene (Figure 5A). Transformed *E.coli* cells that became β gal⁺ contained an open reading frame. In a vector with an amber suppressible termination
5 codon between the 3' end of the cDNA and the 5' end of β gal, the fusion of the cDNA to the β gal was controlled by the Sup phenotype of the *E.coli* strain (Figure 5B). The same type of cloning scheme may be adapted to fuse the 5' end of the M13 phage display protein to the cDNA, in this case viable phage will indicate successful cloning of the open reading frame (Figure 5C).

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Dynamic recoding of the 3' end of the yeast activation domain. The 3' end of the yeast activation domain was recoded to incorporate the controlling elements for *E.coli* gene expression. Figure 6 is one example of recoding the controlling elements needed for bacteriophage T7 protein expression. The recoded yeast activation domain was then used
15 in conjunction with the open reading frame cloning system to fuse the correct reading frame to the activation domain, and simultaneously to a separate 3' fusion protein (for example, β gal or M13gp3).

Phenotypic selection in *E.coli* of cDNA ORFs and concurrent fusion of them to the 3'
20 end of the yeast activation domain and the 5' end of M13gpIII may be performed according to Figure 7: A. Clone random 500 bp cDNA fragments; transform T7RNAP⁺, Sup⁺ *E. coli*; screen plaques. B. Phage Display in *E. coli*. C. Yeast Two Hybrid in Yeast: transform T7RNAP⁺, Sup⁺ *E. coli*; transform M13 into Sup⁻ yeast.

25 The application of which this description and claims form part may be used as a basis for priority in respect of any subsequent application. The claims of such subsequent application may be directed to any feature or combination of features described herein. They may take the form of product, composition, process or use claims and may include, by way of example and without limitation, one or more of the following claims.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Creation of arrayed cDNA libraries. *E. coli* cDNA libraries were plated at a low density (approximately 1000 colonies per plate) onto LB + Amp. Next, 3-4 ml of LB (containing 15% glycerol) was added to each plate and the LB harvested after resuspension of the colonies in the LB was apparent. Plasmid DNA was isolated by means of a kit obtained from Qiagen (Valencia, CA) and used directly to transform the yeast. The transformed yeast were plated onto SD - trp agar plates. The plates were incubated, and the cells harvested as for *E. coli* using 3-4 ml SD - trp + 15% glycerol. The harvested yeast from each plate were separately aliquoted into different wells of deep-dish 96-well plates (the "master library" plates) and frozen at -80°C for long-term storage.

Figure 2. Automatable Y2H format. The following steps were used to perform a Y2H analysis in a microtiter plate: i) Add bait strain to cDNA library strain in a well, ii) allow mating to occur in complex medium, iii) dilute mated mixture into minimal drop-out medium (-leu), iv) allow growth of positively-interacting proteins (growth as readout), v) Perform β Gal assays (quantitative readout), vi) sequence (+) clones, query database(s). Legend: 1 = master library; 2 = daughter; 3 = bait added (mated for 24 - 36 hrs); 4 = selective outgrowth (incubate > 5 days); 5 = archive; 6 = β Gal.

Figures 3A and 3B. Test of reporter activation in a pooled microtiter plate well. Results are expressed as % of interactor in pool of non-interactors. Known interactors were mixed at a known ratio and tested in the liquid mating format against a bait fusion. X axis: mating dilution; y axis: A_{600} or A_{574} . Fig 3A: selective outgrowth after dilution of mated yeast in leucine dropout medium. Activation domain fusion: pJG-scRPB4 (yeast polII subunit); DNA binding domain fusion: pEG-scRPB7 (yeast polII subunit) Fig 3B: β gal assay of wells. Activation domain fusion: pJG-scRPB4 (yeast polII subunit); DNA binding domain fusion: pEG-scRPB7 (yeast polII subunit).

Figure 4. Y2H analysis of the human Nuclear Receptor RXR screened against (approximately) 88×10^4 cDNA clones. Each well of the three 96-well plate shown represents a β gal assay performed on a pEG202 RXR bait plasmid-containing yeast strain mated to approximately 1000 yeast clones of a pJG4.5 AD library. The eight left-most wells in each plate are positive and negative controls, (from top to bottom of the plate); a) pEG202 x pJGRXR, b) pEG202, c) pEGKREV1 x pJGRAF, d) pEGKREV1 x pJGKRIT1, e) pEGRAS x pJGKRIT1, f) pEGRAS x pJGRAF, g) pEGRXR x pJGGOR4, and h) pEGGOR4 x pJGRXR.

Figure 5. Open reading frame cloning strategy. Cloning Open Reading Frames. A. Frameshift fusion to β gal⁺. Randomly sheared cDNA of approximately 600 base pairs were isolated and cloned into a frameshifted β gal gene. Transformed *E.coli* cells that became β gal⁺ contained an open reading frame. B. Frameshift fusion to β gal⁺ in a Sup⁺ host. In a vector with an amber suppressible termination codon between the 3' end of the cDNA and the 5' end of β gal, the fusion of the cDNA to the β gal was controlled by the Sup phenotype of the *E.coli* strain. C. Frameshift fusion to M13gpIII in a Sup⁺ host. The same type of cloning scheme may be adapted to fuse the 5' end of the M13 phage display protein to the cDNA, in this case viable phage will indicate successful cloning of the open reading frame.

Figure 6. Dynamic recoding of the 3' yeast activation domain. Translation of the T7 controlling elements (T7CE). The 3' end of the yeast activation domain (AD) was recoded to incorporate the controlling elements for *E.coli* gene expression. Shown is one example of recoding the controlling elements needed for bacteriophage T7 protein expression. The recoded yeast activation domain was then used in conjunction with the open reading frame cloning system to fuse the correct reading frame to the activation domain, and simultaneously to a separate 3' fusion protein (for example, β gal or M13gp3). Legend: 1 = yeast; 2 = cDNA insert; 3 = frameshift.

Figure 7. Phenotypic selection in *E. coli* of cDNA Open Reading Frames and concurrent fusion of them to the 3' end of the yeast activation domain (AD) and the 5' end of M13gpIII. A. clone random 500 bp cDNA fragments; transform T7RNAP⁺, Sup⁺ *E. coli*; screen plaques. B. Phage Display in *E. coli*. C. Yeast Two Hybrid in Yeast: transform
5 T7RNAP⁻, Sup⁺ *E. coli*; transform M13 into Sup⁻ yeast. Legend: 1 = yeast; 2 = insert site; 3 = frameshift.

CLAIMS

What is claimed is:

1. A method for detecting an interaction between a first test protein and a second test protein, comprising:
 - (a) providing a host cell containing a reporter gene wherein the reporter gene expresses a detectable protein when the reporter gene is activated by an amino acid sequence including a transcriptional activation domain when the transcriptional activation domain is in sufficient proximity to the reporter gene;
 - (b) providing a first chimeric gene that is capable of being expressed in the host cell, the first chimeric gene comprising a DNA sequence that encodes a first hybrid protein, the first hybrid protein comprising:
 - (i) a DNA-binding domain that recognizes a binding site on the reporter gene in the host cell; and
 - (ii) a first test protein or fragment thereof that is to be tested for interaction with at least one second test protein or fragment thereof;
 - (c) providing a second chimeric gene that is capable of being expressed in the host cell, the second chimeric gene comprising a DNA sequence that encodes a second hybrid protein, the second hybrid protein comprising:
 - (i) the transcriptional activation domain; and
 - (ii) a second test protein or fragment thereof that is to be tested for interaction between the first test protein or fragment thereof; wherein interaction between the first test protein and the second test protein in the host cell causes the transcriptional activation domain to activate transcription of the reporter gene;
 - (d) introducing the second chimeric gene into the host cell and subsequently introducing said cells into an arraying means thereby creating a master library plate;
 - (e) introducing cells from the master library plate into a second arraying means thereby creating a mating set;

- (f) introducing the first chimeric gene into the host cell and subsequently introducing said cell into the mating set;
- (g) selecting for outgrowth of the interaction of the first and second genes;
- (h) removing a portion of the mating set to a third arraying means thereby creating a rescue set;
- (i) determining whether the reporter gene has been expressed in the mating set;
- and
- (j) analyzing the cells from the rescue plate.

2. A method for detecting an interaction between a first test protein and a second test protein, comprising:

- (a) providing a host cell containing a reporter gene wherein the reporter gene expresses a detectable protein when the reporter gene is activated by an amino acid sequence including a transcriptional activation domain when the transcriptional activation domain is in sufficient proximity to the reporter gene;
- (b) providing a first chimeric gene that is capable of being expressed in the host cell, the first chimeric gene comprising a DNA sequence that encodes a first hybrid protein, the first hybrid protein comprising:
 - (i) a DNA-binding domain that recognizes a binding site on the reporter gene in the host cell; and
 - (ii) a first test protein or fragment thereof that is to be tested for interaction with at least one second test protein or fragment thereof;
- (c) providing a second chimeric gene that is capable of being expressed in the host cell, the second chimeric gene comprising a DNA sequence that encodes a second hybrid protein, the second hybrid protein comprising:
 - (i) the transcriptional activation domain; and
 - (ii) a second test protein or fragment thereof that is to be tested for interaction between the first test protein or fragment thereof; wherein interaction

between the first test protein and the second test protein in the host cell causes the transcriptional activation domain to activate transcription of the reporter gene;

(d) introducing the second chimeric gene into the host cell and subsequently introducing said cells into an arraying means thereby creating a master library plate;

(e) introducing cells from the master library plate into a second arraying means thereby creating a mating set;

(f) introducing the first chimeric gene into the host cell and subsequently introducing said cell into the mating set; and

(g) selecting for outgrowth of the interaction of the first and second genes.

3. The method according to Claim 1 wherein the host cell is *Saccharomyces cerevisiae*.

4. The method according to Claim 1 wherein the reporter gene is selected from the group consisting of LEU2, lacZ, HIS3, URA3, LYS2, GAL1, *E. coli* galK, GFP, CUP1, CAT, G418 and GUS.

5. The method according to Claim 1 wherein step (f) is performed in liquid media.

6. The method according to Claim 2 in the form of a kit.

7. A method for the selection of a second test protein or fragment as defined in Claim 1, said method comprising:

(a) recoding a yeast activation domain gene, said recoding incorporating DNA controlling elements necessary for *E. coli* gene expression, said DNA controlling elements comprising:

(i) a sequence which acts as an *E. coli* promoter to initiate mRNA transcription;

- ii) a ribosome binding site and an ATG fMet codon necessary to initiate protein translation;
 - iii) a multiple cloning site composed of one or more restriction sites which are preferably unique to the cloning vector, into which to clone stuffer fragments of DNA that can encode protein fusions to the activation domain; and
 - iv) a reporter gene, said reporter gene cloned out-of-frame with respect to the ATG codon;
- (b) fusing the correct reading frame to said activation domain by means of an open reading frame cloning system, said system comprising:
- (i) isolating randomly sheared cDNA of about 600 base pairs;
 - (ii) cloning said base pairs into a frameshifted reporter gene; and
 - (iii) fusing the cDNA to the activation domain; and
- (c) separating clones that restore the reading frame of the reporter gene.

8. The method according to Claim 7 wherein the reporter gene is selected from the group consisting of LEU2, lacZ, HIS3, URA3, LYS2, GAL1, *E. coli* galK, GFP, CUP1, CAT, G418 and GUS.

9. The method of Claim 7 wherein the reporter gene is lacZ.

10. The method of Claim 7 wherein an *E. coli* suppressible termination codon may be encoded between said stuffer fragment and said reporter gene such that in phenotypically suppressing *E. coli* host strains, the stop codon is suppressed by a suppressing tRNA molecule that inserts a specific amino acid.

11. The method of Claim 10 wherein the *E. coli* suppressible termination codon is a TAG amber termination codon.

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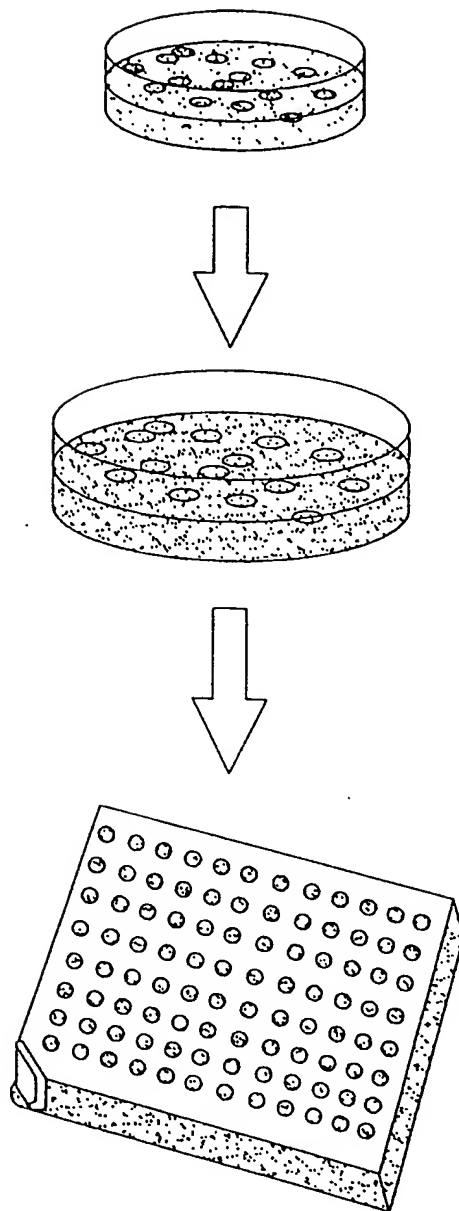


FIG. 1

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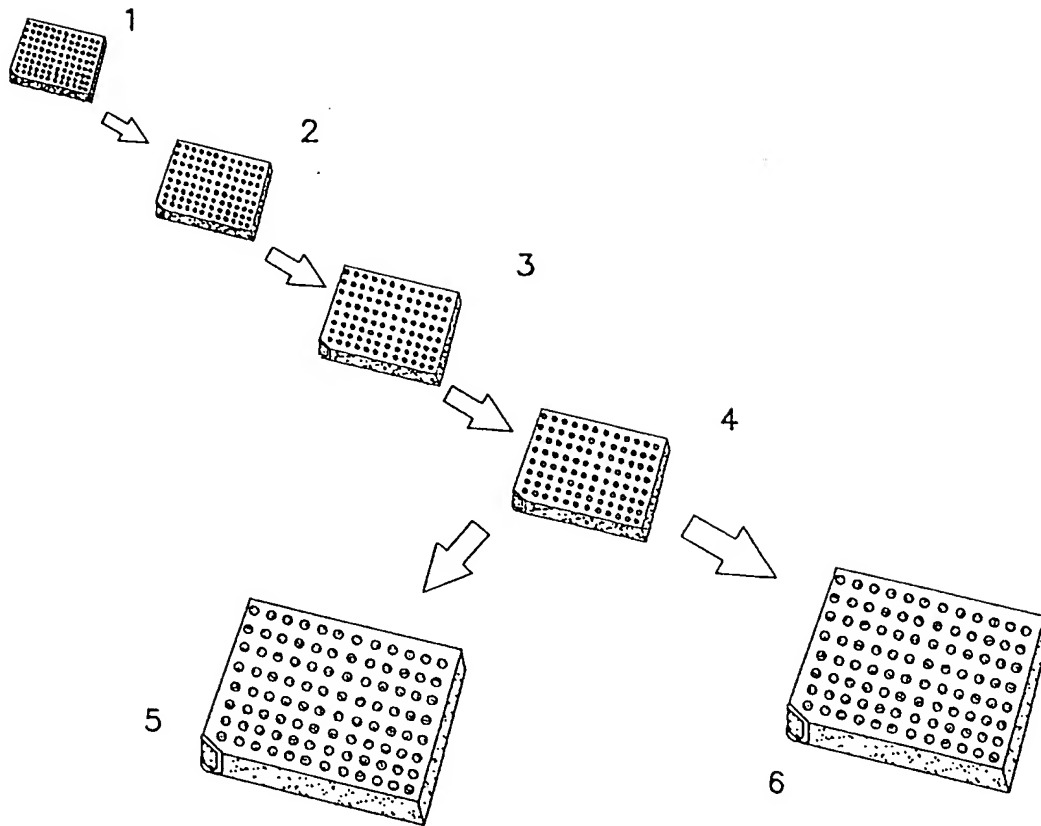


FIG. 2

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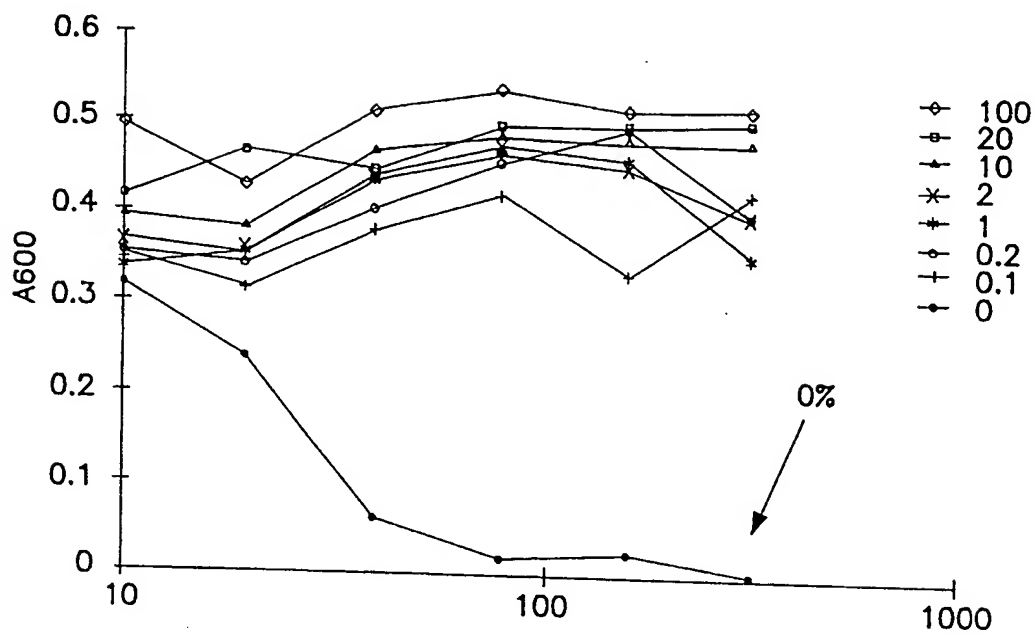


FIG. 3A

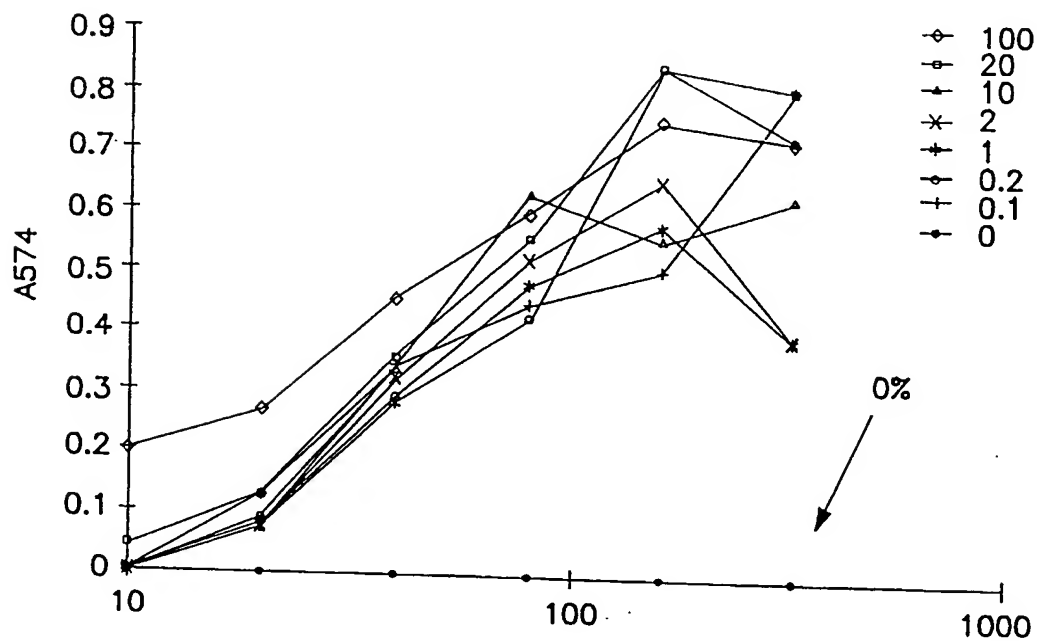


FIG. 3B

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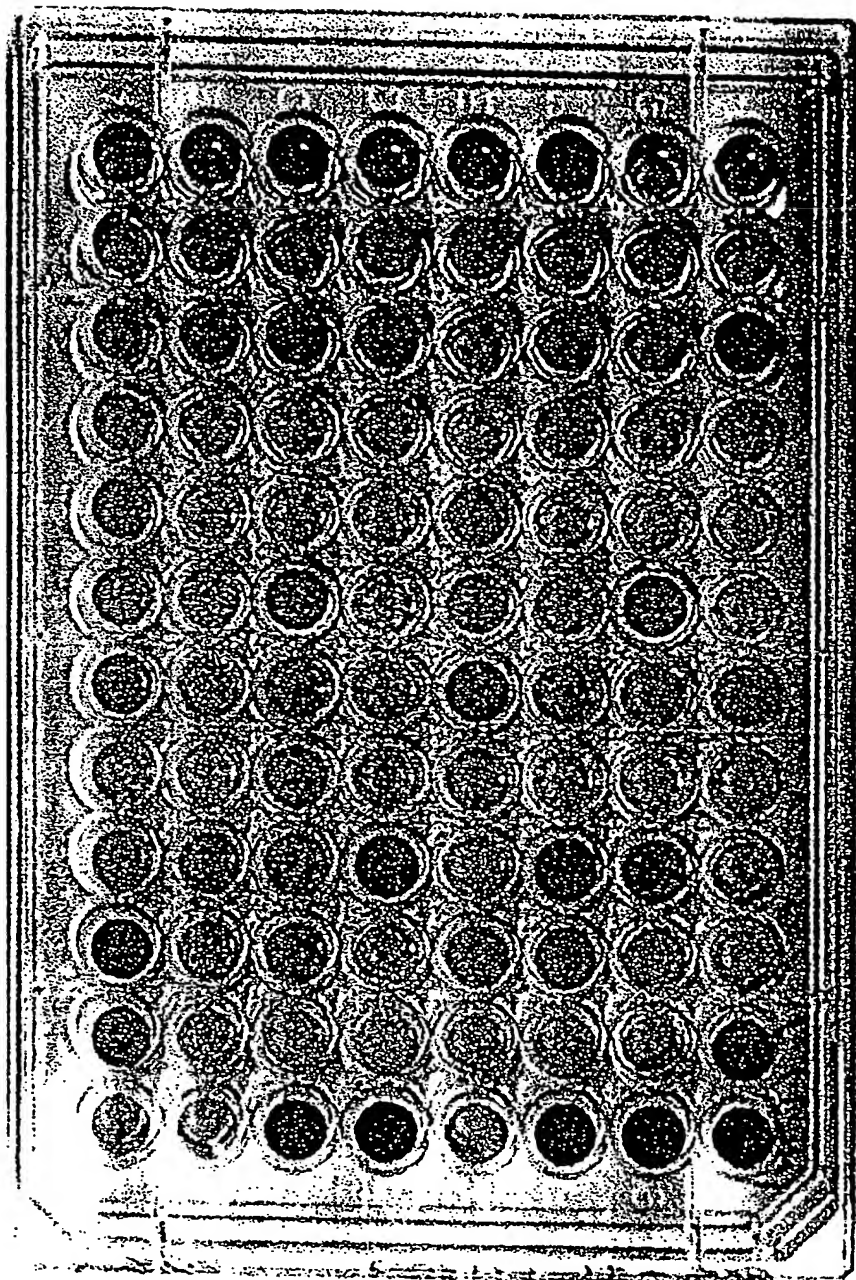


FIG. 4

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FIG. 5A

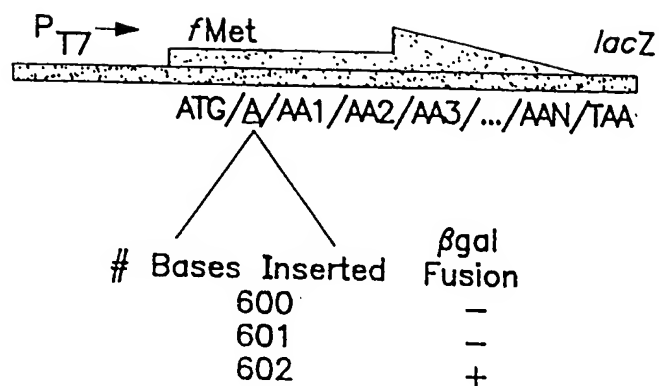


FIG. 5B

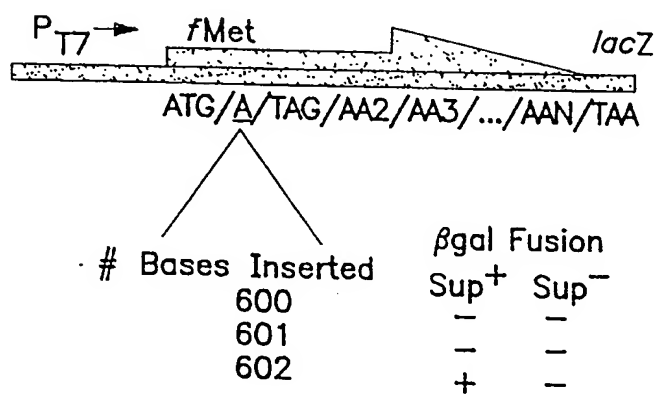
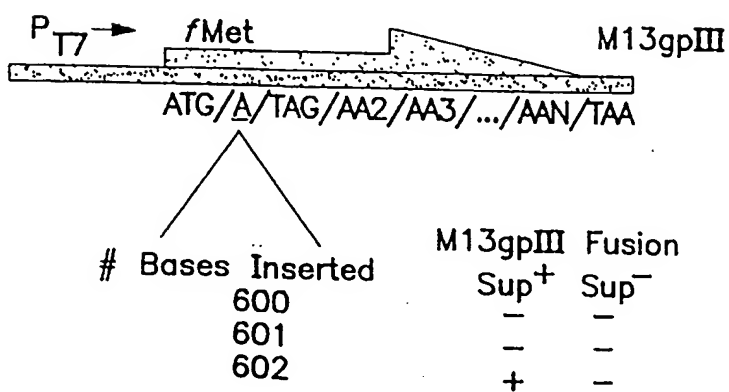


FIG. 5C



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T7 PROMOTER-> RBS fMet...MCS
 TTAATACGACTCACTATAGGGAGACCAAAGAAGGAGATATACATCATG...
 LeuIleArgLeuThrIleGlyArgProLysLysGluIleTyrIleMet...

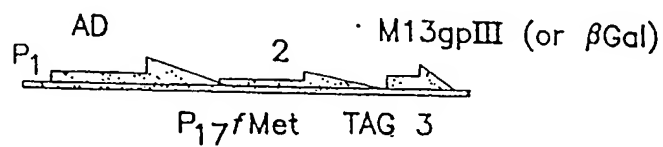
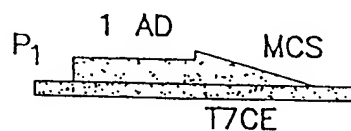


FIG. 6

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FIG. 7A

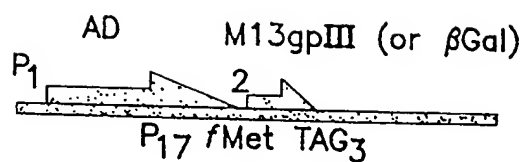


FIG. 7B

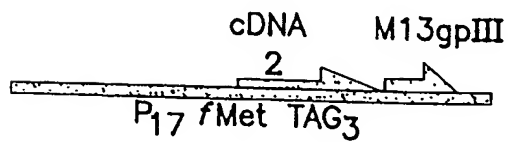


FIG. 7C

